



PATENT

Attorney Docket No. PRMG 04578

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Slater, et al. Group No.: 1652
Serial No.: 09/641,319 Examiner: R. Hutson
Filed: 08/18/2000
Entitled: Thermophilic DNA Polymerases From *Thermotoga Neapolitana*

DECLARATION OF JAMES HARTNETT
UNDER 37 C.F.R. § 1.131

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

CERTIFICATE OF MAILING UNDER 37 C.F.R. 1.8(a)(1)(i)(A)

I hereby certify that this correspondence (along with any referred to as being attached or enclosed) is, on the date shown below, being deposited with the U.S. Postal Service with sufficient postage as first class mail in an envelope addressed to: Commissioner for Patents, P.O. Box 1450, Alexandria, VA 22313-1450.

Dated: 11/12/2003

By: Susan M. McIntock

Examiner Hutson:

I, James Hartnett, hereby declare and state, under penalty of perjury, that:

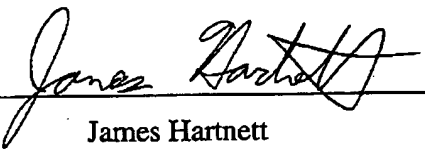
1. I am one of the inventors of the above-named patent application (hereinafter "present application").

2. The claimed invention was reduced to practice in the United States of America prior to January 9, 1995, as indicated from this Declaration and the attached notebook pages. The work presented in the notebook pages was performed in this country by me or under my supervision. As evidenced from these documents, we developed mutant DNA polymerases derived from *Thermotoga neapolitana*. This includes mutants shown in Figure 4 of the present application. For example, the deletion mutant *Thermotoga neapolitana* polymerase labeled as M284, which provides a reduction in 5'-3' exonuclease activity through generation of deletion mutations is described on pages 32 (design) and 50 (activity assay of generated mutant) of the notebook 100893. Likewise, substitution mutants that reduce 3'-5' exonuclease activity were generated. For example, the combination deletion and substitution mutant *Thermotoga*

neapolitana polymerase labeled as M284 (D323A, D389A) in Figure 4 of the present application is described on pages 27 (design) and 54 (use of the mutant enzyme in sequencing reaction) of notebook 100996 (the D323A is referred to as D355A and D389A is referred to as D424A in the notebook).

3. I further declare that all statements made herein are of my own knowledge, are true, and that all statements are made on information and belief that are believed to be true; and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under §1001 Title 18 of the United States Code, and that such willful statements may jeopardize the validity of the application of any patent issued thereon.

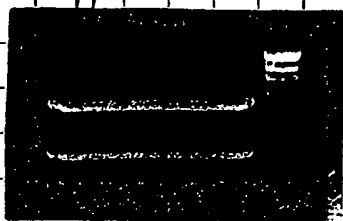
Dated: 11-12-03


James Hartnett

To remove the 5' → 3' exon activity of Tne, our course of action will be to digest the gene at Met 284 with BspHI and ligate that to the NcoI site of pALTERex 1. This removes 283 AAs from the N-terminal end. The BspHI site isn't unique so I have to do some monkeying around.

Digest 1

10 μ l pGTne 1
2 μ l XbaI
2 μ l NdeI
Buffer D



4.2 kb fragment removed and purified with Wizard DNA clean-up.

Digest 2

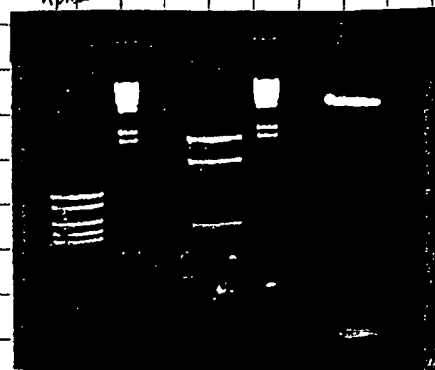
25 μ l 4.2 kb fragment
3 μ l BspHI NEB
2 μ l KpnI
5 μ l Buffer (4) NEB

17 μ l 4.2 kb fragment
2 μ l KpnI
5 μ l Buffer J

4.2 kb
BspHI
KpnI

4.2 kb
KpnI

pALTERex 1
NcoI-XbaI



All fragments seem are of the expected size.

1050 bp BspHI - KpnI pol fragment
1300 bp KpnI - XbaI pol fragment
5846 bp NcoI - XbaI vector

were co-isolated with Wizard and ligated in a 50 μ l volume.

Continued on Page 34

Jim Harshbarger
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Clairie Schenck
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The 5' → 3' *exo* assay consists of incubating two ³²P kinased substrates with 5u of enzyme for one hour at 74°C and then looking for ³²P release off the substrates. Reaction done in duplicate. Release measured by DE81 binding.

Results

As expected Tag and Tne (native) demonstrate a 5' → 3' *exo* activity, whereas UTMa and M284 Tne do not. I don't feel that the 2.7% release from M284 is significant since only one of the two duplicates is showing release.

	SAM	CPM1.	
Ø	1	3880.00	
WASH	2	3978.00	
Ø	3	4016.00	
	4	3966.00	% release
Tag	5	3808.00	
	6	3582.00	7.47%
UTMa	7	4076.00	
	8	3842.00	0%
Tne	9	3478.00	
	10	3552.00	11.9%
M284	11	3998.00	
	12	3768.00	2.77%
	13	3888.00	
	14	4012.00	
Ø	15	3758.00	
	16	3948.00	% release
Tag	17	1170.00	
	18	1132.00	70%
UTMa	19	4060.00	
	20	4044.00	0%
Tne	21	2770.00	
	22	2738.00	28%
M284	23	4074.00	
	24	3992.00	0%

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Jin Hantho
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Elaine Johnson
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PROJECT

Creating the double knock-out mutant

Notebook No.

Continued From Page 24

To be sure 3'→5' ~~is~~ activity is completely removed I want to have a D355A, D424A double mutant. This is accomplished by transferring the 215 bp. Csp45I fragment of D355A into the D424A background.

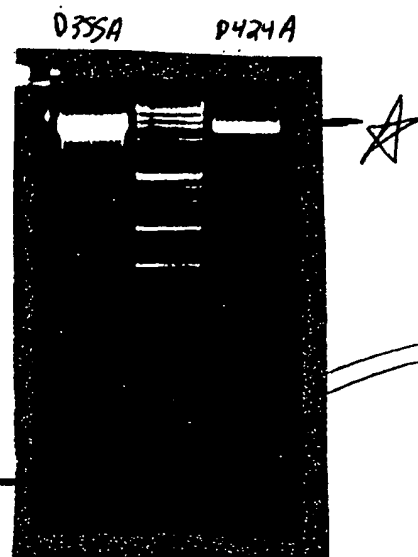
4 µg D355A plasmid cut with Csp45I

1 µg D424A plasmid cut with Csp45I

D424A reaction phosphorylated

run on 1.5% TAE gel

indicated bands co-isolated with Wizard
ligated in 40 µl volume overnight at 16°C



10-18-94 ligation transformed into JM109

10-19-94 8 colonies streaked out

10-20-94 8 mini preps done and cut with BglII

Results

⑤, ⑥, ⑦, + ⑧ show the expected 348bp BglII fragment, although ⑥ seems to have an extra band.



Continued on Page 28

Jim Hartnett

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Clairie Johnson

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I used the following ddNTPs
 Template was Tne mutant TS-5A1 and
 primer was JH66. $70^{\circ} \leftrightarrow 95^{\circ}$.

G = $30 \mu M$
 A = $75 \mu M$
 T = $75 \mu M$
 C = $30 \mu M$

Results

Tne doesn't look much better.
 Taq looks very unbalanced.
 I need to repeat this + try
 lower ddNTPs for A, T, + C.

Taq

Tne

Continued on Page 56

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